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An Analysis of Monoclonal T Cell and Antibody Recognition Sites on Ia Molecules

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The advances made in understanding T cell and antibody recognition sites on Ia using monoclonal helper and alloreactive T cells are summarized. For many antibodies it has been possible to determine whether the antibody recognition site was determined by the α or β chain. Such defined antibody reagents have allowed the definition of multiple functional antigen presenting sites on a given Ia molecule. Mutant antigen-presenting cells independently suggest the existence of such multiple functional sites. A detailed analysis of the I-A^b mutant bm12 directly defines the chemical nature of one such site and suggests that it arose as a result of gene conversion. Such regions of Ia molecules may be important for both T-cell function and antibody-binding.

The nature of antibody or T-cell recognition sites on Ia molecules is poorly defined, even though the complete DNA sequences of several alleles of the Ia polypeptides are known [1–5]. In part this is a reflection of the problems of studying any membrane proteins composed of multiple subunits. One approach to studying antibody sites is to utilize antibodies directed against synthetic peptides and find those antibodies with the desired binding or biological activity. For Ia molecules this approach is .in the preliminary stages. An alternative approach is to try to use existing antibody reagents with known biological activity and characterize their reactivity. We will present data which characterize antibody sites using this approach.

Investigators have utilized several approaches to try to understand the nature of T-cell recognition sites, including naturally occurring mutations [6–9], mutant cell lines selected in vitro [10], mutants constructed using recombinant DNA technology [11–13], and monoclonal antibodies to major histocompatibility complex (MHC) proteins to block MHC recognition by T cells [14–19]. To study Ia function, the sole Ia mutant mouse strain B.6.C-H-2^{bm12} (bm12) has proved invaluable [20–24]. In this paper we will summarize our current understanding of monoclonal antibody and T-cell recognition sites on Ia molecules emphasizing the approaches we have taken using T-cell clones.

We have concentrated on T-cell clones which recognize only "hybrid" antigens, Ia molecules which are unique to F_1 cells [25]. These unique Ia molecules are formed by the combinational association of the α chain from one parent and the β chain from the other parent (Fig 1). For example, one can isolate clones derived from C57BL/6JXA/J (B6XA) F_1 mice which only proliferate in response to the synthetic peptide poly(L-Glu^{60}, L-Ala^{30}, L-Tyr^{10}) (GAT) in the presence of B6XA F_1 cells but not to GAT when presented on either C57BL/6J (B6) or A/J (A) strain cells. We have utilized a large panel of antibodies to characterize a panel of such F_1 restricted T-cell clones. Clones whose proliferation was supported by bm12 × B10.A(4R) F_1 cells and whose proliferation was blocked by anti-I-A^k antibody 10-2.16 recognized $A_\alpha{}^bA_\beta{}^k$ molecules. Clones which were blocked by antibody H116.32 and which did not recognize bm12 × B10.A(4R) F_1 cells utilized a restriction site on $A_\alpha{}^kA_\beta{}^b$ [18,26]. These clones then allow one to try to assess the contribution of 1 Ia chain to both antibody-binding and T-cell recognition.

MONOCLONAL ANTIBODY BINDING CAN BE LOCALIZED TO THE α OR β CHAIN

A direct approach of localizing antibody binding is to isolate the individual α and β chains and simply monitor the precipitation of the isolated material [27]. While this method has the virtue of being the most direct and definite, it is also laborious. Additionally, determinants which are easily destroyed or depend on the presence of another chain, would be lost. Nevertheless, Kupinski, Plunkett, and Freed [27] have mapped 2 monoclonal antibody determinants to the β chain. They were able to assign 10-2.16 and 11-5.2 to the β chain. The monoclonal antibody 10.2.16 had previously been mapped to the β chain [28]. Monoclonal antibodies 40F and 40M also react with the β chain (J. Freed, personal communication). Using isolated chains Kupinski et al [27] were unable to localize either 39F or 39J. However, these investigators were not able to localize any antibody to the α chain.

It is possible to take advantage of the combinational association of the α and β polypeptides of Ia molecules to localize the sites recognized by anti-I-A^k antibodies. This method depends on the observation that one could clearly distinguish by two-dimensional gel analysis all the Ia polypeptides from the *b* and *k* haplotypes and that antibodies used react with the *k* haplotype but not *b* haplotype molecules. The *b* haplotype polypeptide which coprecipitates with the α^k and β^k polypeptides allows one to determine whether a given monoclonal antibody reacted with the α^k or β^k polypeptide. Thus if an antibody reacted with the α^k chain, from ($b \times k$) F_1 extracts it would precipitate 1 α chain and 2 β chains. Conversely if it reacts with a β chain it will precipitate 1 β chain, but 2 α chains (Fig 1). We have done this type of analysis for 8 monoclonal antibodies and the results are presented in Table I [18,26,28].

The final approach to the localization of antibody determinants to the α or β chains is to utilize T-cell clones as typing reagents. Thus one simply monitors the inhibition of proliferation using a collection of clones whose reactivity has been determined previously by their behavior with the bm12 mutation and a panel of monoclonal antibodies whose reactivity has been defined biochemically. Following this reasoning we have

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Abbreviations:
A: A/J
B6: C57BL/6J
B6XA: C57BL/6JXA/J
Bm12: B6.C-H-2^{bm12}
GAT: poly(L-Glu^{60}, L-Ala^{30}, L-Tyr^{10}
MHC: major histocompatibility complex

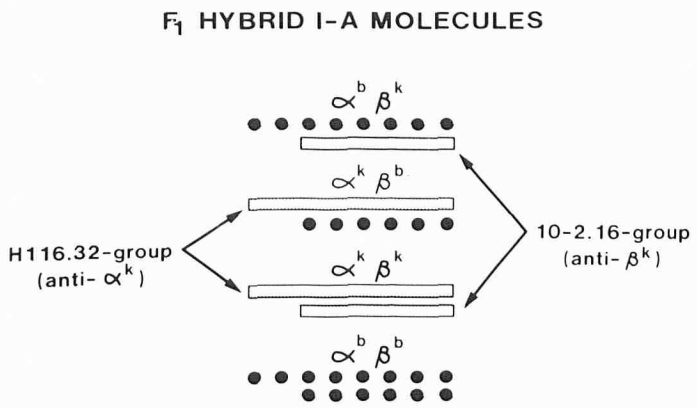

FIG 1. Schematic diagrams of F_1 hybrid I-$A^{b/k}$ molecules showing immunoprecipitation patterns using monoclonal antibodies H116.32 and 10-2.16. H116.32 precipitates A_α^k, $A\beta^k$, and A_β^b polypeptides; 10-2.16 precipitates A_α^k, A_α^b, and A_β^k polypeptides. Reproduced with permission from [26].

TABLE I. *Chain specificities of monoclonal antibodies*

Monoclonal antibody	IA chain recognized	Apparent specificity	Chain Localization Method: Isolated chains	2-D gel coprecipitation	Blocking T cell clones
10.2.16	β^k	Ia 17	+	+	+
10-3-6	β^k	Ia 17	+		
H150-13	β^k	Ia 17			+
40N	β^k	Ia 1		+	+
39E	β^k	Ia 1		+	+
40M	β^k	Ia 1	+[a]	+	+
11-5-2	β^k	Ia 2[b]	+		
40F	β^k	?	+[a]		+
BP107	β^b	?			+
9B	β^b	?			+[c]
39I	β^b	?			+[c]
H116-32	α^k	Ia 19		+	+
39J	α^k	Ia 19		+	+
40J	α^k	Ia 2		+	+
39F	α^k	Ia 2		+	+
H118-49	α^k	Ia 2			+

The determination of Ia chain specificity of monoclonal antibodies (+) indicates the method was used to determine chain specificity. Data is summarized from our unpublished results and [18,26–28]. Monoclonal antibodies isolation and characterization was described previously [29–32].

[a] Personal communication, J. Freed.

[b] The Ia-2 specificity is complex [29] and may be the reason that different antibodies reacting with the Ia-2 specificity map to different Ia chains.

[c] The antibodies also react with I-E molecules.

defined a panel of T-cell clones, one set which recognizes $A_\alpha^k A_\beta^b$ and another set which recognizes $A_\alpha^b A_\beta^k$ (Fig 2). Unknown monoclonal antibodies are thus tested to see if they inhibit the proliferation of one or the other set of T-cell clones. We have performed this type of analysis for all the monoclonal antibodies listed in Table 1 (except 11.5.2). In all the cases where it was possible to compare, (9/9) this method agreed exactly with the biochemical localization [18,26–28]. We have on this basis tentatively mapped two monoclonals which react with both I-E^k and I-A^b molecules to the β^b chain (antibodies 9B and 39I). Obviously this method is indirect for chain localization, but has the advantage of being rapid and relatively simple. Further, it *directly* shows that all these hybrid molecules are in fact utilized by functional T cells.

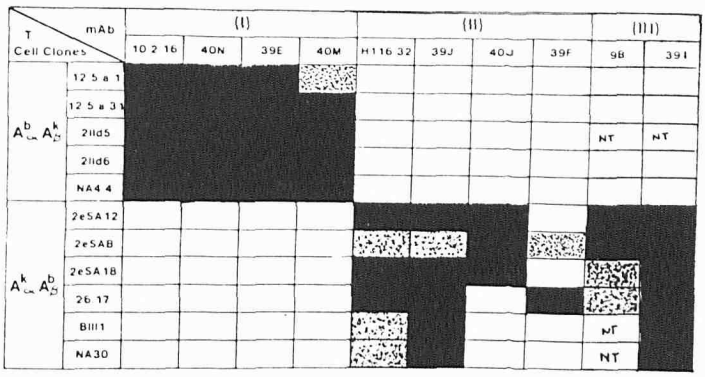

FIG 2. The blocking pattern of monoclonal anti-I-A^k antibodies on antigen induced proliferation of T cell clones as measured by the uptake of [^{3}H]thymidine. Inhibition of T cell proliferation by antibodies has been described previously [18,26]. *Solid boxes* indicate greater than 75% inhibition. *Stippled boxes* indicate 25–75% inhibition. *White boxes* indicate less than 25% inhibition. *NT* indicate not tested. Data compiled from [18,26] and unpublished observations and represent the consensus blocking results from several experiments.

TOPOLOGY OF ANTIBODY-BINDING SITES

In order to gain an insight into the topological relationship of antibody binding sites, competitive binding studies have been performed for many of the antibodies used in our studies [30,33]. In these competitive binding studies, one antibody was radioactively labeled, mixed with unlabeled antibody present in excess, and allowed to react with cells expressing the appropriate Ia molecules. Thus if antibodies reacted with sites identical to or near one another a lower number of counts were bound, i.e., inhibition was observed. Competitive binding studies are consistent with the chain localization assignments in Table I. The competitive binding studies demonstrated that the antibodies we classify as anti-α chain all fall into one cluster while the antibodies we classify as anti-β chain antibodies all fall into another epitope cluster [30]. Another report using other monoclonal antibodies which seemed to react with the same Ia specificities, suggested that Ia-1 and Ia-17 (which we classify as β chain specificities) are close to one another topologically but distant from Ia-2 and Ia-19 (which we classify as α chains specificities) [29] which are in turn close to one another.

bm12 STUDIES DEMONSTRATE MULTIPLE FUNCTIONAL SITES ON Ia

The I-A^b mutant mouse B6.C-H-2^{bm12} and its wild-type counterpart B6 have been used in a variety of studies examining the relationships of immune response genes and Ia function. The bm12 strain retains many of the immune response gene characteristics of the progenitor strain B6, but has altered responsiveness to some antigens [6]. To critically interpret the experiments using this strain it is crucial to know the nature of the bm12 mutation. Serologically and genetically the mutation mapped to the I-A^b subregion of the H-2 complex [20,21]. Peptide map analyses suggested that A_β^{bm12} polypeptide chain was altered while the Aα polypeptide from the bm12 strain was unchanged [34,35]. DNA sequencing of the A_β^{bm12} gene demonstrated 3 nucleotide differences in the NH_2 terminal or first domain when compared to the A_β^b gene [36,37]. These changes were clustered within a stretch of 14 nucleotides corresponding to amino acids 67–71 of the mature β chain polypeptide. This provides a β chain with precisely defined amino acid substitutions for use in functional studies.

We have focused on the ability of the bm12 strain to serve as both an allostimulus for alloreactive T-cell clones and as an antigen presenting cell to antigen reactive T-cell clones. In our

first series of experiments it was found that most of the alloreactive clones which recognized B6 cells did not recognize bm12 stimulator cells. They showed that the bm12 mutation destroyed the allostimulation site recognized by these clones. However, 1 clone recognized *both* bm12 and B6 cells, implying that there are at least 2 allorecognition sites on the I-A^b molecule. Even more surprisingly, among clones specific for the $A_\alpha{}^bA_\beta{}^k$ hybrid molecule, 2 clones were found which recognized bm12 cells, although neither recognized B6 cells. All of the other F_1-restricted clones did not recognize either B6 or bm12 cells. One of these clones is an alloreactive A anti-B6XA F_1 clone, while the other is a GAT-reactive clone of B6XA origin. The existence of these two different classes of clones suggests that there are at least 2 sites recognized by T-cell sites on the $A_\alpha{}^bA_\beta{}^k$ molecule [38]. Hochman and Huber also find similar evidence for multiple functional sites on Ia molecules using the bm12 mutant and B6 strains and monitoring their ability to present different insulins to primed T cells [39].

Whether or not the sites are precisely where the mutation occurs or in fact are at a distant site whose conformation is influenced by the mutation is unknown. However, it is clear that this mutation is critical to the formation of the site recognized by T cells.

MONOCLONAL ANTIBODIES DEMONSTRATE MULTIPLE FUNCTIONAL SITES

Antibodies have been used to study Ia recognition by T cells. The advent of T-cell cloning and monoclonal antibodies has allowed a detailed system in which to study this interaction. As an example we have used a panel of antibodies to inhibit a panel of F_1 restricted T cell clones. Antibodies 10.2.16 and H116-32 distinguished 2 mutually exclusive families (Fig 2). One group of clones was inhibited by 10.2.16 and not by H116-32 (Group I) and the other group exhibited reciprocal inhibition (Group II). Within the H116-32 group (Group II) the pattern was complex. In particular, monoclonal antibodies 40J and 39F were clearly different. In this type of analysis one might argue that overlapping or subsetting patterns of inhibition of T-cell clone proliferation might simply reflect differences in the relative affinity of antibodies and T cells for Ia molecules. However, this cannot explain the differential blocking seen with monoclonals 40J and 39F. These differences suggested that there were multiple sites on the $A_\alpha{}^kA_\beta{}^b$ molecule [26]. A similar analysis using Anti-I-E reactive monoclonals and T-cell clones show the same type differential inhibitory effects of monoclonal antibodies. Thus, these results suggest that there are multiple functional T-cell sites on the I-E^k molecule [40].

Studies with I-A^k mutant cell lines selected in vitro with antibodies suggest that there are multiple functional sites on the I-A^k molecule [10]. In these studies some I-A^k restricted T-cell clones were able to respond to antigen on both the mutant and parent cell lines while others could only respond to the parent line. This indicates that one site was destroyed by the mutation while the other site was unaffected. In addition, such studies suggested that certain determinants recognized by antibodies tended to be coordinately lost, consistent with our assignments to the α or β chains [10].

HYPERVARIABLE REGIONS MAY FUNCTION IN ANTIGEN PRESENTATION

One very interesting finding which arose from the sequencing of the I-A α and I-E β genes was that the allelic differences were clustered [5,41]. Different strains of mice, which expressed different alleles, tended to vary in the same regions. Using the analogy to immunoglobulins these were termed hypervariable regions [5]. Such regions may also exist for the A βchains, though there is less sequence data and such regions are not as well defined [3]. Previously, the ability to respond to a variety of defined antigens (Ir genes) was mapped in to the I-A or I-E subregions, by utilizing recombinant strains of mice which expressed different Ia alleles [19]. Therefore, it was speculated that these "hypervariable" regions were critical for determining immune responsiveness. A prediction of such a model would be that alterations in such regions would alter immune responsiveness in vivo and in vitro.

THE bm12 MUTATION MAY BE THE RESULT OF A GENE CONVERSION EVENT

One striking example of how a functional site can be treated and analyzed arose from the study of the bm12 mouse. As mentioned previously, the entire sequence of the bm12 I-A chain is known. Because of the nature of the substitutions, it has been proposed that these differences arose from a gene conversion event involving the $A_\beta{}^b$ and $E_\beta{}^b$ gene [36,37,42]. The 3 amino acids where $A_\beta{}^{bm12}$ differs from $A_\beta{}^b$ are identical to those found in $E_\beta{}^b$. These substitutions correspond to one of the so-called hypervariable regions of the E polypeptide [41,42]. Even more striking is the finding that some T cells restricted to I-E region molecules, recognize bm12. Hochman and Huber found that B6 mice, which normally do not express I-E molecules, did not respond to sheep insulin while other strains of mice expressing $E_\beta{}^k$ or $E_\beta{}^b$ did. Surprisingly, the mutant bm12 strain also responded and could present sheep insulin to primed T cells suggesting that T cells recognized a part of $A_\beta{}^{bm12}$ which "looked like" $E_\beta{}^b$ [39]. We have described an alloreactive T cell clone, which is restricted to the I-E region which recognizes bm12 cells but not B6 cells [42]. These experiments provide direct functional evidence that the bm12 mutation arose from a gene conversion event and that this event is immunologically relevant. Further, the recognition of this region by an alloreactive T-cell clone provides direct evidence of the importance of "hypervariable" regions in T-cell stimulation. Thus not only class I [43] but class II [36,37,39,42] products can be altered by gene conversion events.

Gene conversion thus may serve as a mechanism which acts on class Ia genes to create sequence diversity over evolutionary time. If in fact each hypervariable region can independently function as "antigen binding" or interaction sites, the presence of such multiple sites increases the number of ways Ia can present antigen to T cells.

ANTIBODY BINDING SITES

One can correlate the binding of monoclonal antibodies with the amino acid sequence predicted by cDNA sequencing studies by the following scheme. Using the monoclonal antibodies defined as reacting with the α or β chains one can take advantage of the strain distribution pattern of the monoclonal antibodies reactivity. In effect, the strains expressing different I-A alleles served as "mutants" though they are complex in that there are multiple differences between the alleles. The most DNA sequence data are available for the I-A αchains so we examined the strain distribution pattern of monoclonals 40J, 39F, 116-32, and 39J. These antibodies all react with *k* haplotype strains and not the *d*, *b*, *f*, *u*, and *q* haplotype strains. Thus as our first approximation, we looked for residues present in the *k* haplotype but not in the *b*, *d*, *f*, and *u* haplotypes. There are 2 such regions, at amino acid positions 57 and 65, and we suggest that these regions are important for binding of these antibodies. Intriguingly these sites are in the "hypervariable" regions of the A_α chains which may be important in antigen presentation. It is important to note this type of analysis in this case neglects the contribution of the β chain. However, we nonetheless feel it is an important first step. For example, not only can one directly test by transfection and site-directed mutagenesis the prediction that these sites are important in antibody binding and T-cell function, but one can specifically predict exactly which changes should alter those biologic activities.

Ultimately one would like to know the nature of T-cell

restriction or functional sites, the determinants recognized by antibodies, and the structural basis of these sites. A final resolution of these questions may ultimately lie in detailed x-ray crystallographic analysis of these molecules. However, the studies summarized here represent important initial steps in characterizing in molecular terms the sites of Ia molecules recognized by T cells and antibodies.

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